Histol Histopathol (2007) 22: 651-660

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Protein-energy malnutrition alters histological and ultrastructural characteristics of the bone marrow and decreases haematopoiesis in adult mice

J.G. Xavier[1], M.E. Favero[2], M.A.R. Vinolo[3], M.M. Rogero[4], M.L.Z. Dagli[5], V.E. Arana-Chavez[6], R. Borojevic[7] and P. Borelli[3]

[1]Faculty of Veterinary Medicine, Methodist University of Sao Paulo, São Bernardo do Campo, SP, Brazil, [2] Haematology Laboratory, Department of Pathology and Clinical and Toxicological Analyses, University of State of Londrina, Brazil, [3]Experimental Haematology Laboratory, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Brazil, [4]Biochemistry of Nutrition Laboratory, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Brazil, [5]Department of Pathology, Faculty of Veterinary Medicine and Zootechny, University of Sao Paulo, Brazil, [6]Laboratory of Mineralized Tissue Biology, Department of Histology and Embryology, Institute of Biomedical Sciences, University of Sao Paulo, Brazil, [7]Institute of Biomedical Sciences and Program of Applied Cellular Biology Medicine, Hospital Universitario Clementino Fraga Filho, Federal University of Rio de Janeiro, Brazil

Summary. Protein-energy malnutrition (PEM) decreases resistance to infection by impairing a number of physiological processes, including haematopoiesis. The aim of this study was to evaluate the microanatomical aspects of bone marrow (BM) in mice that were subjected to PEM, in particular, with respect to the components of the local extracellular matrix and the proliferative activity of haematopoietic cells. For this, histological, histochemical, immunohistochemical and ultrastructural techniques were used. Two-month old male Swiss mice were fed with a low-protein diet containing 4% protein and control mice fed a 20% protein diet. When the experimental group had attained a 25% loss of their original body weight, we collected the different biological samples. Malnourished mice had presented severe BM atrophy as well as a reduction in proliferating cell nuclear antigen and gelatinous degeneration. The malnourished mice had more fibronectin accretion in paratrabecular and endosteal regions and more laminin deposition in perisinusal sites than controls. Endosteal cell activation and hyperplasia were found, suggesting their participation in the process. Additionally, we have observed a decrease in the capacity of malnourished haematopoietic stroma to support the growth of haematopoietic stem cells ($CD34^+$) *in vitro*. These findings point to a structural impairment of the haematopoietic microenvironments in mice with PEM, possibly hampering the interactions between cells and cellular signalling.

Key words: Gelationous degeneration, Protein-energy malnutrition, Extracellular matrix, Bone marrow, Haematopoiesis, Microenvironment

Introduction

The four most incident nutritional deficiency diseases in developing countries are protein-energy malnutrition (PEM), xerophthalmia, nutritional anaemias, and iodine deficiency disorders. Of these, PEM is the most frequent and the most difficult to control. The primary cause of PEM is usually an insufficient intake of food or proper utilization of energy and protein (Latham, 1990). The haematopoietic tissue, like all the tissues that have a high rate of cell renewal and proliferation, requires an ample supply of nutrients, and may thus be altered by deficient nutritional states. It has been well established, in other studies (Chandra, 1991, 1992, 1997; Chandra and Kumani, 1994; Borelli et al., 1995; Krenitsky, 1996; Schrimshaw and SanGiovanni, 1997; Woodward, 1998), that PEM

Offprint requests to: Primavera Borelli, Experimental Haematology Laboratory, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Prof. Lineu Prestes, 580 - B17, CEP 05508-900, Sao Paulo, SP, Brasil. e-mail: borelli@usp.br

Abreviations. BM: Bone marrow, ECM: Extracellular matrix, FN: Fibronectin, LN: Laminin, PCNA: Proliferating Cell Nuclear Antigen, PEM: Protein energy malnutrition, PG: Proteoglycans, TEM: Transmission electron microscopy,

modifies both the specific and non-specific immune response to an infectious agent, as well as impairing haematopoiesis. Of all the effects that PEM has on immunocompetence, the most striking are: i) atrophy of the lymphoid tissue, particularly in the thymus; ii) a reduction in delayed cutaneous hypersensitivity; iii) a reduction in the number of T cells, especially T helper cells; iv) a decrease in thymulin activity; v) hindering of secretory immunoglobulin A antibody response; vi) a reduced concentration and activity of complement components and phagocyte dysfunction (Redmond et al., 1991; Chandra, 1992; Woodward et al., 1992; Ozkan et al., 1993; Borelli and Nardinelli, 2001). The exact mechanisms underlying these alterations have not yet been elucidated.

The bone marrow (BM) is a physiologically dynamic tissue that can present an increased cell turnover rate and a wide variation in cellularity. In normal conditions (Wilkins, 1992), 25% to 75% of the bone marrow cavity is filled with haematopoietic cells in different stages of maturation and stromal cells (reticular cells, macrophages, endothelial cells and adipocytes), in addition to extracellular matrix (ECM) proteins and cytokines (Weiss, 1986; Valli, 1991; Wilkins, 1992). The composition of the bone marrow ECM is topographically variable and is associated with the development of different lineages of blood cells, suggesting the existence of specific interactions between stem cells and stromal elements (Nilsson et al., 1998, 2001).

Severe PEM imposes a variety of effects on all cells of the body. This has been evidenced by the report of diminishment or loss of cell proliferation in several organs following severe PEM (Suda et al., 1976; Betancourt et al., 1989; Borelli et al., 2004). Cell proliferation has been shown to be decreased in the bone marrow and spleen of malnourished mice, which also presented a lower number of pluripotent progenitors when compared to control mice (Fried et al., 1978). A study carried out to determine the effect of malnutrition on the cell cycle in bone marrow cells reported a decrease in the number of viable nucleated cells and a decrease in the mitosis index (Olmos et al., 2001). In previous studies, we demonstrated that the production of cells of the myeloid lineage is hampered in PEM (Borelli et al., 1995) and that ECM proteins are shown to be quantitatively altered (Vituri et al., 2000, 2005) which may account for the bone marrow atrophy and pancytopenia observed in subjects with PEM.

The aim of this study was to characterise the microanatomical aspects of the bone marrow and their correlation with haematopoiesis *in vivo* and *in vitro* using mice that were subjected to PEM.

Materials and methods

Diets

The murine diets were prepared in our laboratories (Table 1). Mineral and vitamin mixtures were prepared according to the recommendations for adult mice of the American Institute of Nutrition (Reeves et al., 1993). The control diet contained 20% (w/w) protein whereas the hypoproteic diet only 4% (w/w). The source of protein used was casein. Except for the protein content, the two diets were identical and isocaloric, the total amount of casein removed from the formulation of the hypoproteic diet having been substituted for the same mass of corn starch. The final protein content of each ration was monitored by the standard micro-Kjeldahl method (Instituto Adolfo Lutz, 1967).

Mice

Male, outbred, Swiss and C57BL/6J mice, 2 to 3 months old, were obtained from the Faculty of Pharmaceutical Sciences at the University of São Paulo. They were placed in individual "metabolic cages", and received the control diet for 21 days. After this adaptation period, the mice were subsequently divided into two groups and maintained under a regular light/dark cycle of 12 h, temperature of 22-25°C and a relative humidity of 55±10%, receiving either the control or the low-protein diet and water *ad libitum*. Their body weight was monitored every 72 h and the consumption of food every 48 h. Mice were submitted to experimental assays when the undernourished group lost 25% of their original body mass (Borelli et al., 1995). For the collection of the different biological samples, the mice were previously anesthetized with xylazine (10 mg/kg) (Rompum“, Bayer, Brazil) and ketamine chlorohydrate (100 mg/kg) (Ketamina“, Cristália, Brazil). The Commission for Ethics in Animal Studies of the Faculty of Pharmaceutical Sciences at the University of São Paulo approved this study.

Blood

Heparinized blood samples were obtained via cardiac puncture and collected concomitantly with the bone marrow cells and sternum for histological,

Table 1. Composition of experimental diets[1].

Ingredients	Control group	Malnourished group
	(g/kg diet)	
Casein (>85% protein)	200	40
Sucrose	100	100
Fiber	10	10
Corn oil	80	80
Mineral mixture[2]	40	40
Vitamin mixture[2]	10	10
L-Methionine	1.5	1.5
Choline bitartrate	2.5	2.5
Cornstarch	556.5	716.5

[1]: Isocaloric diets providing 1716.3 kJ/100g (410.6kcal/100g); [2]: Mineral and vitamin mixtures were prepared according to the 1993. Recommendations of the American Institute of Nutrition for adult mice (Reeves, 1993).

immunohistological and ultrastructural studies. Total and differential blood cell counts were carried out (Lecoq, 1972). Plasma was separated by centrifugation and the total protein content and albumin was determined by the standard methods used in medical analysis (Lecoq, 1972).

Bone marrow: cells

Bone marrow cells were obtained by flushing the femoral cavity with McCoy's 5A medium (Sigma Chemical Co., St. Louis, MO, USA). They were quantified in a hemocytometer, and cytocentrifuge smears were stained by the standard May-Grunwald and Giemsa solutions (Sigma Chemical Co., St. Louis, MO, USA). Differential cell counts were performed considering 500 cells per animal.

Bone marrow: histology and morphometry

We studied the sternal bone marrow. The sternum was removed and immediately immersed in Carnoy fixative for 1 hour, and processed by standard histological techniques (paraffin-embedding). For immunohistochemistry, the sternum was fixed for 1 hour in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA), decalcified in 5% nitric acid for 3 hours and paraffin-embedded. Sternum sections, 5 mm thick, were stained by Hematoxillin-Eosin (HE), Masson's Trichrome, Picrosirius, Gordon & Sweets (reticulin), Periodic Acid Schiff (PAS) and Alcian Blue pH 2.5. Picrosirius-stained sections were studied under polarised light. Morphometry was done using a computerised image analysis system (BIOSCAN/ OPTIMAS®).

Immunohistochemistry: Fibronectin, Laminin and Proliferating Cell Nuclear Antigen (PCNA)

For immunohistochemical analyses, BM sections were labelled with the following primary antibodies: polyclonal anti-fibronectin (1/400 dilution), polyclonal anti-laminin (1/25 dilution), and anti-PCNA PC10 clone (1/100 dilution), all purchased from DAKO, Denmark. Immunostaining was performed according to a streptavidin-biotin peroxidase technique using the StreptABComplex-HRP Duet System – DAKO, Denmark. The immunoreactivity was visualized after incubation with freshly prepared 0.5% 3.3 diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) and 0.045% hydrogen peroxide in PBS, pH 7.2. Slides were counterstained with Harris' hematoxillin. The endogenous peroxidase of BM tissue was blocked with 3% hydrogen peroxide for 30 minutes in the dark previous to exposure to primary antibodies. Negative controls were carried out omitting the primary antibody.

Processing for transmission electron microscopy (TEM)

Fragments of sternum (1 mm^2) from which all the adjacent soft tissues were removed were immersed in 2% glutaraldehyde (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 2.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 mol/L cacodylate buffer, pH 7.4 (Sigma Chemical Co., St. Louis, MO, USA) for 6 h at room temperature and left overnight at 4°C (Arana-Chavez and Katchburian, 1995). Specimens were decalcified in 4.13% EDTA (Sigma Chemical Co., St. Louis, MO, USA), pH 7.2 for 7 days, extensively washed in 0.1 mol/L cacodylate buffer, pH 7.4, and post-fixed in 1% cacodylate-buffered osmium tetroxide (Sigma Chemical Co., St. Louis, MO, USA) for 2 h. Specimens were dehydrated in graded concentrations of ethanol and in acetone before embedding in Spurr® (Electron Microscopy Sciences, Fort Washington, PA, USA) at 70°C for 72 h. Toluidine blue-stained 1 mm thick sections were examined by light microscopy, and regions containing the bone marrow and the adjacent endosteal surface were trimmed for ultra thin sectioning. Sections (80 nm) were cut with a diamond knife on a Leica Ultracut R ultramicrotome® (Leica, Buffalo, NY, USA), collected onto 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined under a Jeol 1010® transmission electron microscope (Jeol, Tokyo, Japan) operated at 80 kV.

Processing for ultrastructural cytochemistry

The evaluation of the presence of proteoglycans (PG) in the bone marrow ECM was made using a cytochemical staining of sternum fragments with the cationic dye ruthenium red, for the ultrastructural identification of PG (Minguell and Tavassoli, 1989; Arana-Chavez and Katchburian, 1995). However, at this point we have not yet carried out the enzymatic treatment of the samples, and therefore have not characterised the chemical nature of the PG molecules.

Bone fragments were collected as previously described and fixed in the same fixing agent for 36 h. Specimens were washed in a 0.1 mol/L sodium cacodylate buffer, pH 7.4, containing 0.1% ruthenium red, and processed as described later. Some specimens were left unstained before examination, which was carried out in a Jeol 1010 transmission electron microscope, for better identifying the electron opaque deposits corresponding to ruthenium red.

Long-term mouse bone marrow cultures

The long-term bone marrow assays were carried out according to the protocol described by Spooncer et al. (1993). 2 to 3 month old C57BL/6J mice were used for this experiment. The mice were submitted to the same procedures described above to achieve malnutrition. Long-term bone marrow cultures (LTBMC) are able to sustain haematopoiesis without the necessity of adding growth factors, for these are supplied by the stromal cells that form a cellular and an extracellular matrix. The capability of this system to sustain haematopoiesis is totally dependent on the establishment of a layer of

adherent cells that are derived from the stroma of the bone marrow (Spooncer et al., 1993). This type of assay can only be carried out using cells of isogenic animals, the C57BL/6J strain being the one most frequently used in studies involving this type of assay.

The cells from control (C) and malnourished (M) mice from both femora and tibiae were flushed out with Fisher's medium (Sigma Chemical Co., St. Louis, MO, USA) and supplemented with 10% of horse serum (Cultilab, Campinas, Brasil). After collection, the cells were quantified in a hemocytometer using trypan blue (Sigma Chemical Co., St. Louis, MO, USA) and were plated in 24 well culture plates ($2x10^5$ cell/well) in Fisher's medium supplemented with 25% of horse serum (Cultilab, Campinas, Brasil), 1 μmol/L hydrocortisone sodium succinate (Sigma, St. Louis, MO, USA), 100,000 UI/L of penicillin and 100 mg/L of streptomycin (Sigma Chemical Co., St. Louis, MO, USA). The cultures were maintained at 33°C with a 5% CO_2 atmosphere. The cultures were fed fresh growth medium every 7 days. After the establishment of an adherent layer of cells (which took almost 1 month) the cultures were irradiated to ablate endogenous haematopoieses with a dose of 5 Gy delivered by a caesium-137 source at a dose rate of 3.52 Gy/min. The old medium was removed and $CD34^+$ cells obtained from BM of control mice were plated to the stroma ($5x10^4$ cell/L). $CD34^+$ cells were isolated by using anti-mouse CD34 RAM 34 clone (Pharmyngen, BD Biosciences, USA) and mouse anti-rat kappa microbeads (Miltenyi Biotec, Germany), followed by magnetic column enrichment according to the manufacturer's instructions (Miltenyi Biotec, Germany). The establishment of the stroma and consequent haematopoiesis were evaluated weekly using an inverted microscope.

Statistical analysis

The dependent variables are normally distributed. Results were analysed using the t test, and results were considered significant at $\alpha \leq 0.05$ throughout this study.

Results

From the amount of nitrogen present in the diets, we calculated that the control diet contained 18.0±1.4% (n=3) of protein whereas the hypoproteic diet contained 4.0 ± 0.6% (n=5) of protein.

The mice that were maintained on the hypoproteic diet spontaneously reduced their ration consumption up to 31.9% in relation to control mice, which resulted in the decrease of protein consumption, body weight loss and decrease in plasma concentrations of protein and albumin (Table 2). The malnourished group experienced a 25% loss of body weight in a period of 14 to 16 days, after the introduction of the hypoproteic diet.

Blood and bone marrow cellularity

The malnourished mice were anaemic (haemoglobin concentration: control = 13.25±0.38 g/dL; malnourished = 10.34±0.45 g/dL, $P \leq 0.05$) with reticulopenia (control = 6.96±0.87%; malnourished = 1.20±0.21%, $P \leq 0.05$) and depletion of polymorphonuclear granulocytes, lymphocytes and monocytes (Table 3). Differential counts of BM cells indicated a significant decrease in the number of polymorphonuclear cells, macrophages and erythroblasts (Table 3). The malnourished mice presented a significant decrease in the number of cells of all maturation steps of the granulocytic lineage, associated to a significant decrease of the erythrocytic compartment, which was clearly detected not only by the reduced number of erythroid cells, but also by an expressive increase in the granulocytic:erythroid and lymphoid:erythroid ratios (Table 3). These results characterise marrow failure, most likely due to the impairment of primitive BM cell progenitors.

Bone marrow: morphometry and histology

The malnourished mice (n=4) presented shrinkage of the marrow haematopoietic space with a decrease of the bone marrow area occupied by cells (15.73±2.03%) in comparison to the control mice (Fig. 1A) (52.89±2.78%, n=3, $P \leq 0.05$), as well as presenting myeloid atrophy in which there was depletion of erythroid and granulo-monocytic compartments and, in more severe cases of malnutrition, also of the megakaryocytic compartment (Fig. 1B). The bone marrow sinuses appeared to be dilated and interstitial areas became filled with slightly acidophilic granular material, characterising a gelatinous degeneration of the bone marrow, with associated local haemorrhage. Alcian Blue and PAS were positive in these regions (data not shown), suggesting an increase in

Table 2. Food and protein intakes, body weight variation and plasma protein and albumin concentration of control and malnourished mice.

Groups	n	Food Intake (g/day/animal)	Protein intake (g/day/animal)	Body weight variation (% initial weight)	Plasmatic Protein (g/dL)	Plasmatic Albumin (g/dL)
Control	15	3.57±0.63	0.71±0.12	2.30±1.77	7.20±0.98	4.20±0.60
Malnourished	35	2.43±0.81*	0.10±0.03*	-25.00±3.88*	4.80±0.80*	3.10±0.40*

The results are shown as mean values plus or minus standard deviation. Asterisks indicate when there was significant difference between the experimental and control groups ($P \geq 0.05$).

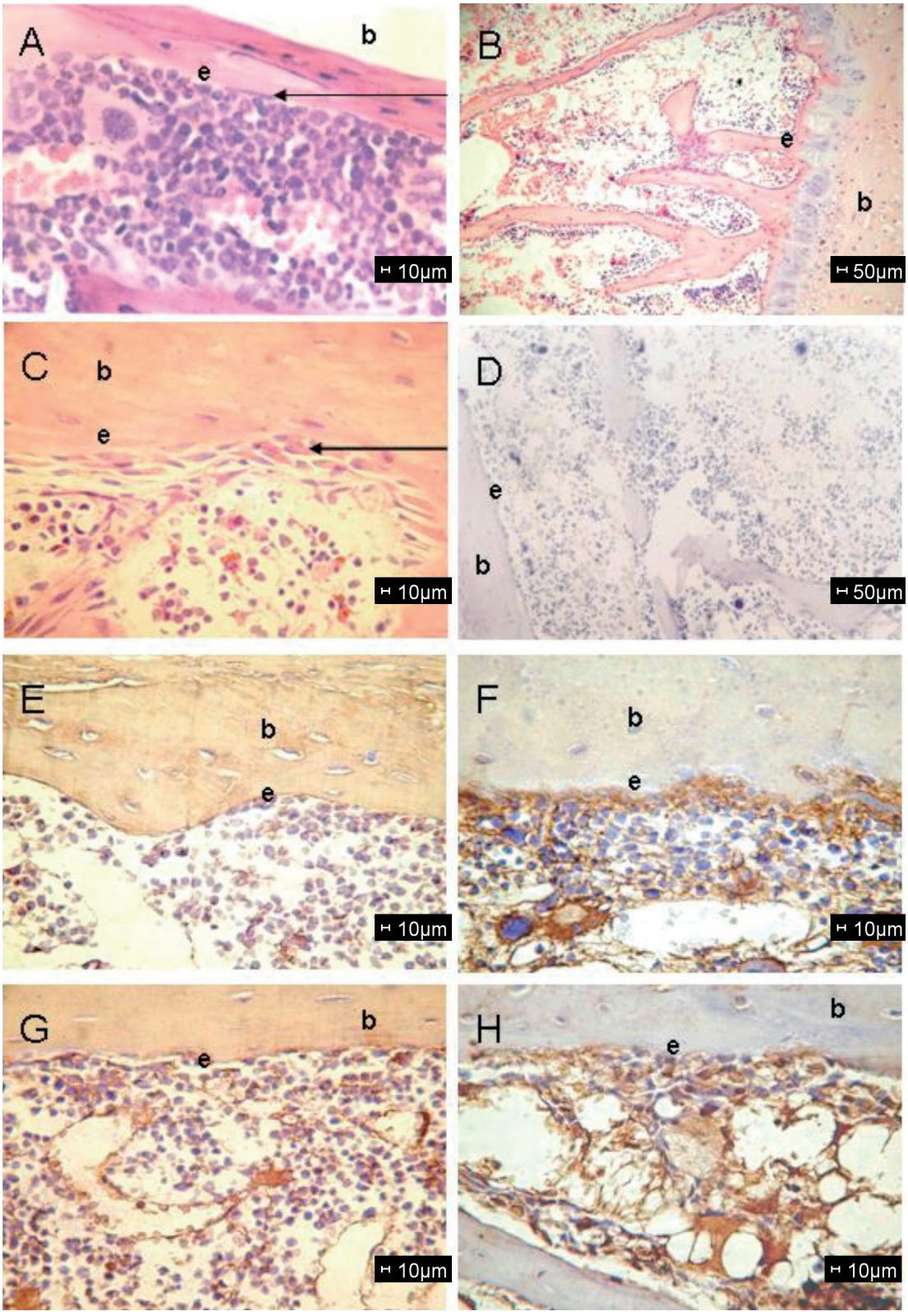

Fig. 1. Sections are of bone and marrow interface showing the bone (b), endosteal regions (e) and the central marrow region. **A.** Bone marrow biopsy section from a control animal showing normal cellularity and the endosteal area (Arrow). Embedded in paraffin (HE stain, x 400). Representative section of control (n=5) and malnourished group (n=15). **B.** Bone marrow biopsy section from a malnourished animal showing severe atrophy. Embedded in paraffin (HE stain, x 100). Representative section of control (n=5) and malnourished group (n=15). **C.** Bone marrow biopsy section from a malnourished animal showing areas of stratification (Arrow) and eventual projection into bone marrow cavity. Embedded in paraffin (HE stain, x 400). Representative section of control (n= 5) and malnourished group (n= 15). **D.** Negative control used in the immunohistochemical analyses. Embedded in paraffin (x 100). **E, F, G, H.** Bone marrow biopsy section labeled with specific antibodies against the extracellular matrix protein fibronectin from a control **(E)** and a malnourished animal **(F)** and with antibodies against laminin from a control **(G)** and a malnourished animal **(H)**. Embedded in paraffin (x 400). Control (n=4) and malnourished group (n=5).

the amount of ECM. The endosteal cells presented anisocytosis with architectural dearrangement, as well as areas of stratification and occasional projection into the BM cavity (Fig. 1C). In these regions, staining by Alcian Blue was increased. Using polarized light analysis of picrosirius-stained slides, a shift from an intense red birefringence in control mice (data not shown) to a weakly yellow colour in the malnourished ones could be observed, indicating a loss of collagen density.

Bone marrow: distribution of fibronectin and laminin

An increase in the accretion of fibronectin (FN), mainly in endosteal/paratrabecular sites of the sternum, was observed in the BM of malnourished mice (Fig. 1E,F). The malnourished mice also presented an increase in laminin (LN) accretion, particularly in perisinusoidal regions of the marrow (Fig. 1G,H). These results are in accordance with our quantitative analyses (Vituri et al., 2000).

Proliferating Cell Nuclear Antigen (PCNA) expression

The immunostaining of PCNA positive cells yielded a dark brown deposit in the nuclei. The proliferative index was expressed as the percentage of labelled cells per 3000 nucleated cells. We analysed the number of positive cells considering three distinct regions of the BM: the subendosteal, paratrabecular and perisinusoidal regions. The malnourished mice (n=8) presented a significant decrease in the number of cells expressing PCNA (8.57±3.59%) in comparison to the control mice (20.18±5.34%, n=7, $P \leq 0.05$) despite the region analysed, which is evidence of a decline in BM cell proliferation.

Bone marrow: ultrastructural analysis

Bone marrow cells were in close proximity to one another in all the control specimens, with ECM restricted to marrow interstitial areas. The endosteum was a continuous layer of flattened bone lining cells that were apposed to the osteoid (Fig. 2A). Conversely, in the experimental group, the areas occupied by the ECM were larger, and some bone marrow cells presented morphological sign of suffering (irregular cytoplasmic boundaries and debris) (Fig. 2B). The endosteum showed signs of greater activity when compared to the control group, as was evidenced by a layer of newly formed bone over the pre-existing bone, and in the majority of cases, a typical cement line was clearly discerned separating the two layers of bone (Fig. 2C,D).

The ultrastructural cytochemical analysis showed electron opaque deposits with a granular appearance, which corresponded to proteoglycan complexes stained by ruthenium red. The control specimens exhibited small deposits uniformly dispersed between the bone marrow cells. They were better observed when unstained ultra thin sections were examined, and appeared forming larger accumulates in close relation to the plasma membrane of the cells (Fig. 3A). The specimens of undernourished mice appeared to contain larger proteoglycan complexes. They constituted a clearly discernible network and filled the larger intercellular spaces between the bone marrow cells (Fig. 3B). Higher magnification revealed larger clumps in close apposition to the cell membranes (Fig. 3C).

Long- term bone marrow culture

The stroma obtained from BM cells of malnourished mice did not adequately sustain haematopoiesis. The stroma obtained from the control mice established itself 3 to 4 weeks after plating, and the adhering layer was predominantly made up of cells with fibroblastoid characteristics, adipocytes, and frequent macrophagic cells. On the other hand, the stroma obtained from malnourished mice only established itself later (5 to 6 weeks after plating), and in several samples did not establish itself at all. The emergence of haematopoietic cells occurred already in the first week after the plating

Table 3. Blood, bone marrow cells and bone marrow ratios of control and malnourished mice

BLOOD	Control (cells/mm^3)	Malnourished (cells/mm^3)
Erythrocytes (x10^6)	9.43±0.24	6.03±0.24*
Leucocytes	3158±408	845±154*
Neutrophils	1045±270	176±21*
Lymphocytes	1914±249	662±97*
Monocytes	83±23	7±3*
BONE MARROW	(x10^6/femur/animal)	(x10^6/femur/animal)
Total cell count	9.81±1.10	4.76±0.32*
Blast cells	0.50±0.08	0.02±0.03*
Promyelocytic and myelocytic cells	0.60±0.09	0.35±0.02*
Band cells	2.08±0.25	1.11±0.15*
Segmented cells	1.80±0.29	1.30±0.14*
Eosinophils	0.16±0.04	0.07±0.01*
Erythroblasts		
Proerythroblast+Basophilic	0.09±0.01	0.02±0.01*
Polychromatophilic	0.58±0.08	0.08±0.01*
Orthocromatic	0.62±0.01	0.02±0.00*
Lymphocytes	1.50±0.15	1.58±0.07
Macrophages	0.09±0.02	0.04±0.02*
Plasma cells	0.01±0.00	0.00±0.00*
BONE MARROW RATIOS		
Granulocytic/erythroid	3.41±0.02	22.50±0.07*
Granulocytic/lymphoid	2.93±0.15	1.71±0.07*
Lymphoid/erythroid	1.16±0.01	13.20±0.15*

Results, expressed as mean plus or minus standard deviation of the total number of erythrocytes and leucocytes, and total number of cells, as well as the number of cells belonging to the different haematopoietic lineages present in the bone marrow of control mice (n=15) and malnourished mice (n=35). Asterisks indicate when there was significant difference between the experimental and control groups ($P \leq 0.05$).

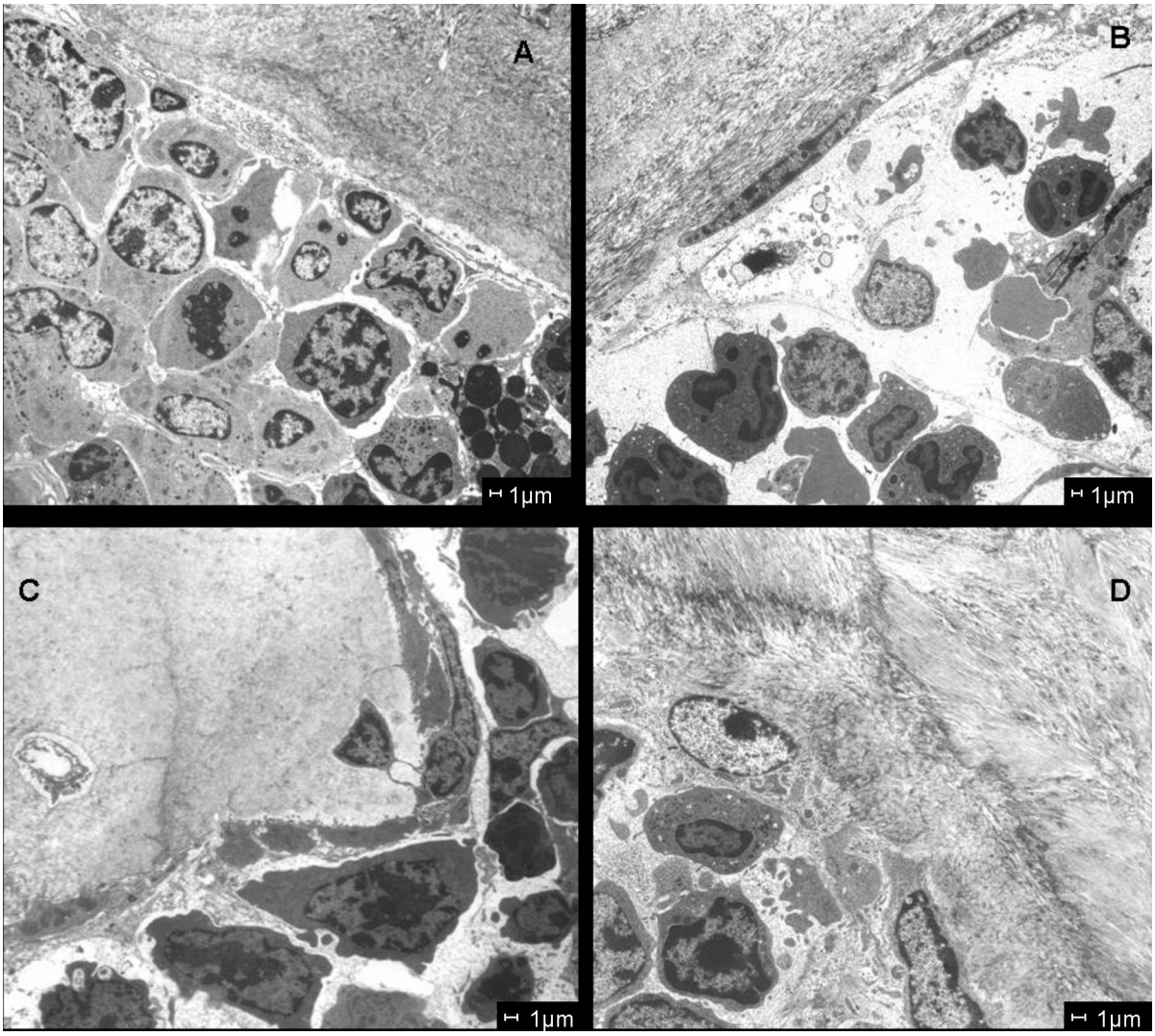

Fig. 2. Electron micrograph showing a part of the bone marrow adjacent to the endosteum. Uranyl acetate/lead citrate staining. **A.** Control group (x 2400). **B, C, D**. Experimental group. Note that abundant extracellular matrix appears between the cells (B, x 2000). Note that a new layer of bone has been deposited over the pre-existing bone surface. A cement line is observed at the boundary between the two regions of bone. In the new forming bone, a new osteocyte appears connected to the outer osteoblasts by two fine cell processes (C, x 2700). Electron micrograph (D, x 2750) showing the endosteal surface of bone at a region different to that shown in C. A new layer of bone has been deposited over the preexisting bone from which it is separated by a cement line. Representative sections of control (n=2) and malnourished groups (n=2).

Table 4. Results of the Long term bone marrow cultures (LTBMC).

	Clusters		Colonies		"Cobblestone" areas	
Time (wk)	Control	Malnourished	Control	Malnourished	Control	Malnourished
1	430±11	430±52	14±1	10±2*	0	0
2	77±20	125±40	81±7	28±8*	7±2	1±1*
3	73±16	41±11*	68±12	45±11*	10±1	3±1*
4	71±19	48±7*	71±4	42±9*	4±1	0*
5	76±9	57±5*	78±13	49±13*	4±2	1±1*
6	87±6	57±12*	61±14	37±10*	8±2	4±1*
7	56±3	55±19	41±11	23±4*	8±2	2±1*
8	79±20	69±15	57±22	28±7*	8±3	2±1*
9	90±4	55±4*	75±14	32±10*	10±4	2±1*

Results, expressed as mean plus or minus standard deviation of the total number of clusters, colonies and "cobblestone" areas counted in the long term BM culture of control (n=4) and malnourished (n=4) mice. Asterisks indicate when there was significant difference between the experimental and control groups ($P \leq 0.05$).

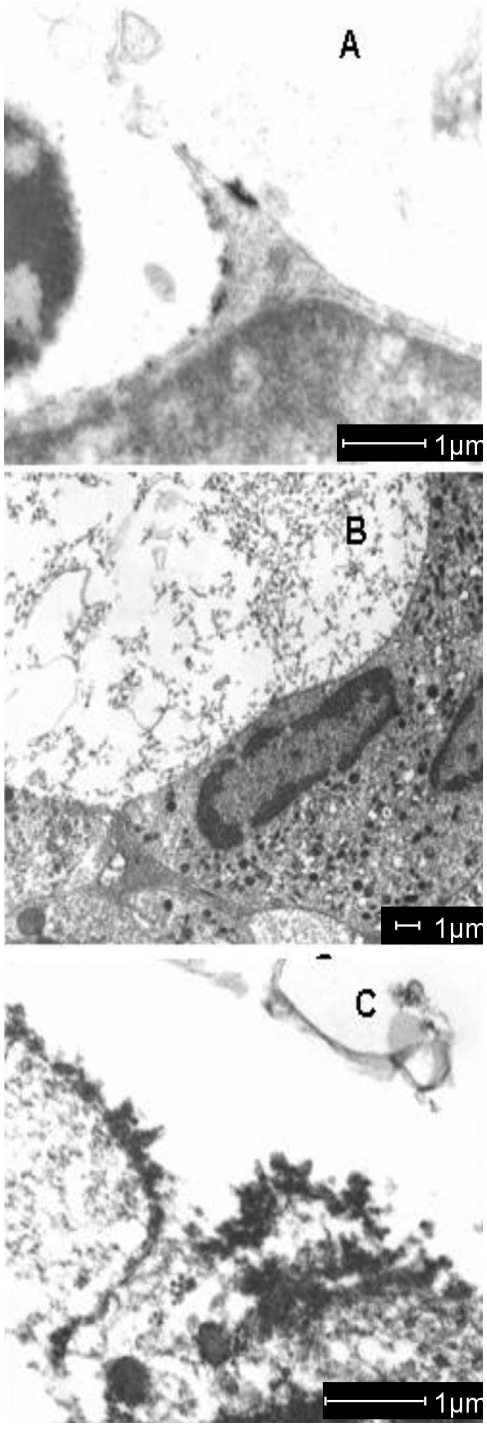

Fig. 3. Ultrastructural cytochemistry using ruthenium red. **A.** Control group (n=2). Electron micrograph showing an ultrathin section that was left unstained for better discerning the electron opaque deposits corresponding to the cationic dye. Note that while small deposits of the dye appear interspersed along the extracellular matrix, some of them accumulate on the plasma membrane of a cell (x 34000). **B.** Experimental group (n=2). Electron micrograph showing abundant electron opaque deposits corresponding to the cationic dye interspersed along the extracellular matrix (x 9600). **C.** Electron micrograph showing large electron opaque accumulates corresponding to the cationic dye in close relation to the plasma membrane of a cell (x 35000)

of $CD34^+$ cells in both groups alike. However, the number of clusters (clusters are groups of cells made up of 2 to 49 cells that originated from a single cell) and colonies (colonies refers to groups of 50 cells or more. In these assays, it is assumed that both clusters and colonies are derived from a single cell) and the "cobblestone" areas throughout the culture were significantly smaller in the stroma originating from malnourished mice (Table 4). The cultures persisted up to 10 weeks after the plating of $CD34^+$ cells and in the stroma of the control mice there was a persistence of areas of haematopoiesis, whilst these disappeared in the stroma from malnourished mice.

Discussion

This study has found that PEM produces both qualitative and quantitative alterations on both the stromal component and the extracellular matrix of the bone marrow, inducing a decrease in haematopoiesis which contributes to the anaemia and leucopoenia found in PEM. However, due to the decrease in the consumption of ration presented by the malnourished group, it can not be determined whether the changes in the bone marrow ECM were exclusively due to protein deficiency or if they resulted from the general stress response that accompanies a large decrease in feed intake.

The gelatinous degeneration (also known as serous atrophy, mucoid degeneration and starvation marrow) of the bone marrow is a condition that is characterised by hypoplasia of haematopoietic cells in the bone marrow and deposition of focal or generalized extracellular gelatinous substances, especially hyaluronic acid (Cornbleet et al., 1977; Seaman et al., 1978). The gelatinous degeneration has been observed in chronic disorders, such as chronic infections, neoplasia, malnutrition and anorexia nervosa. In the present study, we found the bone marrow of malnourished mice to bear a condition similar to that of gelatinous degeneration observed in humans.

The altered BM microenvironment potentially modifies haematopoiesis, both *in vivo* and *in vitro*. Only a few studies correlate PEM with ECM alterations. Lyra et al. (1993) observed an increase of the ECM density in the thymus, and considered this to be responsible for the depletion of thymocytes. In a previous study, we reported an increase in the extractable fibronectin and laminin from bone marrow in mice with PEM (Vituri et al., 2000).

In the present study, we found an atrophic state with subversion of the haematopoietic environment, which may hamper the spatial relationship between haematopoietic and stromal cells, as well as disturb components of the ECM. These data complement and shed new light on one of our earlier studies (Vituri et al., 2000), in which we observed an impact on the terminal differentiation of BM cells induced by PEM. The endosteal lining, which predominantly contains flattened cells organised in a single layer, occasionally substituted

by osteoblasts and osteoclasts (Wickramasinghe, 1997), was heterogeneous, with irregular cell orientation and form, projected into the marrow cavity and occasionally becoming cuboid, with larger nuclei and a reticular chromatin (Fig. 2B). These characteristics are suggestive of cell activity, and confer to the endosteum a reactional aspect, similar to the aspect observed during foetal development or even in marrow/bone injury and repair (McManus and Weiss, 1984; Islam et al., 1990, 1992; Brach and Herrmann, 1991). In this study, cement lines marking the border of pre-existing bone and the new bone layer was observed on TEM (Fig. 4C,D) indicating an increase in the activity of endosteal cells. The expansion of the ECM around endosteal cells in the BM of malnourished animals was evidenced by Alcian blue, suggesting a local accretion of acidic proteoglycans. There is evidence that these molecules influence cell-cell and cell-matrix interactions suggesting that they have important roles in controlling haematopoiesis (Kolset and Gallagher, 1990).

A pre-requisite for normal haematopoiesis is the location of stem cells in specific microenvironments, which allows for interactions between cells and the ECM (Tavassoli and Minguell, 1991). The proper location of stem cells is dependent upon migration within the marrow followed by a selective retention in specific endosteal niches (Nilsson et al., 2001).

The interaction between primitive progenitor cells and the stroma depends upon the presence of proteoglycans secreted by stromal cells (Siczkowski et al., 1992), which can interact with other components of the ECM such as fibronectin, participating in the regulation of cell proliferation (Minguell and Tavassoli, 1989). Although only ruthenium red staining without enzymatic treatment was used, the ultrastructural cytochemical data suggest that the quantity of sulphated proteoglycans is different in malnourished animals. Whilst a normal distribution of fibronectin and laminin has been described in the BM (Nilsson et al., 1998), references describing alterations of these proteins in malnutrition were not found. In addition to finding an increase in acid proteoglycans we describe an increase in the accretion of LN and FN in the bone marrow of malnourished mice, particularly in endosteal and subendosteal sites. The immunohistochemical evidence of the intense accretion of FN in malnourished animals suggests a possible alteration of the interaction between FN and its receptors in the adhesion and migration of haematopoietic cells (Gordon, 1988; Tavassoli and Minguell, 1991; Clark et al., 1992). Haematopoietic stem cells express two receptors for FN: VLA-4 and VLA-5, and there is evidence that these receptors may modulate, independently, the adhesion and migration of already committed primitive and progenitor cells (Giet et al., 2002). Considering that the molecules of the ECM are involved in the regulation of adhesion, migration, proliferation and differentiation of the haematopoietic cells (Mayani et al., 1992; Klein, 1995), such alterations can interfere in the processes of growth and differentiation of haematopoietic cells (Borelli et al., 1995; Klein, 1995; Davidson and Benn, 1996; Vituri et al., 2000). Accordingly, results obtained in this study demonstrate that the presence of PCNA was significantly reduced in the cells of mice with PEM, suggesting that alterations of the BM microenvironment may be responsible for the alteration in cell proliferation (Betancourt et al., 1995; Gonzalez et al., 2002).

In addition to the modifications found *in vivo*, studies in which haematopoiesis was attempted *in vitro* evidence that the stroma obtained from malnourished animals is less capable of supporting haematopoiesis after co-culture with $CD34^+$ cells obtained from control animals, indicating that the stromal cells obtained from malnourished animals present functional alterations.

The BM atrophy found in PEM, as well as the anaemia and leukopenia present in peripheral blood suggest a hampering of the proliferative capacity of progenitor cells, once the decrease of the marrow compartment was not due to the accelerated efflux of cells to the peripheral blood.

Biochemical, structural and cellular alterations of the haematopoietic microenvironment consequent to PEM may contribute to the BM atrophy observed in this state.

Acknowledgements. The authors thank PhD Marinilce Fagundes dos Santos for their suggestions in the course of this work; and Miss Dulce M. Schimieguel, Mr. Gaspar F. de Lima and Miss Sabrina S. Tessi for their technical assistance.

References

Arana-Chavez S.A.M. and Katchburian E. (1995). Junctions between early developing osteoblasts of rat calvaria as revealed by freeze-fracture and ultrathin section electron microscopy. Arch. Histol. Cytol. 58, 285-292.

Betancourt M., Ortiz R., Gomes J.L., Hernandez M.E. and Cravioto J. (1989). Effect of renutrition on cellular proliferation and SCE in bone marrow cells from malnourished rats. Nutr. Rep. Int. 40, 959-964.

Betancourt M., Ortiz R., Gonzalez C., Perez P., Cortes L., Rodríguez L. and Villasenor L. (1995). Assessment of DNA damage in leukocytes from infected and malnourished children by single cell gel electrophoresis/comet assay. Mutat. Res. 331, 65-77.

Borelli P., Blatt S.L., Rogero M.M. and Fock R.A. (2004). Haematological alterations in protein malnutrition. Rev. Bras. Hematol. Hemoter. 26, 49-56.

Borelli P., Mariano M. and Borojevic R. (1995). Protein malnutrition: effect on myeloid cell production and mobilization into inflammatory reactions in mice. Nutr. Res. 15, 1477-1485.

Borelli P. and Nardinelli L. (2001). Protein-calorie malnutrition: decrease in the peritoneal macrophage's respiratory burst. Braz. J. Pharm. Sci. 37, 51-60.

Brach M.A. and Herrmann F. (1991). Hematopoietic growth factors: interactions and regulation of production. Acta Haematol. 86, 128-137.

Chandra R.K. and Kumari S. (1994). Nutrition and immunity: an overview. J. Nutr. 124, 1433S-1435S.

Chandra R.K. (1991). Nutrition and immunity: lessons from the past and new insights into the future. Am. J. Clin. Nutr. 53, 1087-1101.

Chandra R.K. (1997). Nutrition and the immune system: an introduction. Am. J. Clin. Nutr. 66, 460S-463S.

Chandra R.K. (1992). Protein-energy malnutrition and immunological responses. J. Nutr. 122, 597-600.

Clark B.R., Gallagher J.T. and Dexter T.M. (1992). Cell adhesion in the stromal regulation of haemopoiesis. Baillieres Clin. Haematol. 5, 619-652.

Cornbleet P.J., Moir R.C. and Wolf P.L. (1977). A histochemical study of bone marrow hypoplasia in anorexia nervosa. Virchows Arch (Pathol Anat) 374, 239-247.

Davidson J.M. and Benn S.I. (1996). Regulation of angiogenesis and wound repair. In: Cellular and molecular pathogenesis. Sirica A.E. (ed). Lippincott-Raven. Philadelphia. pp 79-107.

Fried W., Shapiro S., Barone J. and Anagnost A. (1978). Effect of protein deprivation on hematopoietic stem cells and on peripheral blood counts. J. Lab. Clin. Med. 92, 303-310.

Giet O., Van Bockstaele D.R., Di Stefano I., Huygen S., Greimers R., Beguin Y. and Gothot A. (2002). Increased binding and defective migration across fibronectin of cycling hematopoietic progenitor cells. Blood 99, 2023-2031.

Gonzalez C., Najera O., Cortes E., Toledo G., Lopez L., Betancourt M. and Ortiz, R. (2002). Hydrogen peroxide-induced DNA damage and DNA repair in lymphocytes from malnourished children. Environ. Mol. Mutagen. 39, 33-42.

Gordon M.Y. (1988). Extracellular matrix of the marrow microenvironment. Br. J. Haematol. 70, 1-4.

Instituto Adolfo Lutz. (1967). Normas de qualidade para a alimentação; vol.1, São Paulo.

Islam A., Glomski C. and Henderson E.S. (1990). Bone lining (endosteal) cells and haematopoiesis: a light microscopic study of normal and pathologic human bone marrow in plastic-embedded sections. Anat. Rec. 227, 300-306.

Islam A., Glomski C. and Henderson E.S. (1992). Endothelial cells and haematopoiesis: a light microscopic study of fetal, normal, and pathologic human bone marrow in plastic-embedded sections. Anat. Rec. 233, 440-452.

Klein G. (1995). The extracellular matrix of the hematopoietic microenvironment. Experientia 51, 914-926.

Kolset S.O and Gallagher J.T. (1990). Proteoglycans in haemopoietic cells. Biochim. Biophys. Acta 1032, 191-211.

Krenitsky J. (1996). Nutrition and the immune system. A.A.C.N. Clin. Issues 7, 359-369.

Latham M.C. (1990). Protein-energy malnutrition - its epidemiology and control. J. Environ. Pathol. Toxicol. Oncol. 10, 168-180.

Lecoq R. (1972). Manuel d'Analyses Médicales et de Biologie Clinique. Doin. Paris.

Lyra J.S, Madi K., Maeda C.T. and Savino W. (1993). Thymic extracellular matrix in human malnutrition. J. Pathol. 171, 231-236.

Mayani H., Guilbert L.J. and Janowska-Wieczorek A. (1992). Biology of the hemopoietic microenvironment. Eur. J. Haematol. 49, 225-233.

McManus P.M. and Weiss L. (1984). Busulfan-induced chronic bone marrow failure: changes in cortical bone, marrow stromal cells, and adherent cell colonies. Blood 64, 1036-1041.

Minguell J.J. and Tavassoli M. (1989). Proteoglycan synthesis by hematopoietic progenitor cells. Blood 73, 1821-1827.

Nilsson S.K., Debatis M.E., Dooner M.S., Madri J.A., Quesenberry P.J., and Becker P.S. (1998). Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. J. Histochem. Cytochem. 46, 371-377.

Nilsson S.K., Johnston H.M. and Coverdale J.A. (2001). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cells niches. Blood 97, 2293-2299.

Olmos S., Reinoso M.F., Marquez M.G. and Roux M.E. (2001). Cytogenetic studies in bone marrow cells from Wistar rats in protein malnutrition. Metabolism 50, 1025-1029.

Ozkan H., Olgun N., Sasmaz E., Abacioglu H., Okuyan M. and Cevik N. (1993). Nutrition, immunity and infections: T lymphocyte subpopulations in protein—energy malnutrition. J. Trop. Pediatr. 39, 257-260.

Redmond H.P., Leon P., Lieberman M.D., Hofmann K., Shou J., Reynolds J.V., Goldfine J., Johnston R.B. Jr and Daly J.M. (1991). Impaired macrophage function in severe protein-energy malnutrition. Arch. Surg. 126, 192-196.

Reeves P.G., Nielsen F.H. and Fahey G.C.Jr. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 132, 1939-1951.

Schrimshaw N.S. and SanGiovanni J.P. (1997). Synergism of nutrition, infection, and immunity: an overview. Am. J. Clin. Nutr. 66, 464S-477S.

Seaman J.P., Kjeldsberg C.R. and Linker A. (1978). Gelatinous transformation of bone marrow. Hum. Pathol. 9, 685-92.

Siczkowski M., Clarke D. and Gordon M.Y. (1992). Binding of primitive hematopoietic progenitor cells to marrow stromal cells involves heparan sulfate. Blood 80, 912-919.

Spooncer E., Eliason J. and Dexter T.M. (1993). Long-Term mouse bone marrow cultures. In: Haemopoiesis: a practical approach. 1st ed. Testa N.G. and Molineux G. (eds). Oxford University Press. New York, NY. pp 55-74.

Suda A.K., Mathur M., Deo K. and Deo M.G. (1976). Kinetics of mobilization of neutrophils and their marrow pool in protein-calorie deficiency. Blood 48, 865-75.

Tavassoli M. and Minguell J.J. (1991). Homing of hemopoietic progenitor cells to the marrow. Proc. Soc. Exp. Biol. Med. 196, 367-373.

Valli V.E.O. (1991). The hematopoietic system. In: Pathology of domestic animals. 4th ed. Jubb K.V.F., Kennedy P.C. and Palmer N. (eds). Academic Press. Orlando. pp 101-265.

Vituri C.L., Alvarez-Silva M., Trentin A.G. and Borelli P. (2000). Alterations in proteins of bone marrow extracellular matrix in undernourished mice. Braz. J. Med. Biol. Res. 33, 889-895.

Vituri C.L., Alvarez-Silva M., Trentin A.G., Tramonte V.L.C.G. and Borelli P. (2005). Thrombospondin in protein malnutrition induced hypoplasia. Rev. Nutr. 18, 727-731.

Weiss D.G. (1986). Histopathology of canine noneoplastic bone marrow. Vet. Clin. Pathol. 15, 7-11.

Wickramasinghe S.N. (1997). Dyserythropoiesis and congenital dyserythropoietic anemias. Br. J. Haematol. 98, 785-797.

Wilkins B.S. (1992). Histology of normal haemopoiesis: bone marrow histology. J. Clin. Pathol. 45, 645-649.

Woodward B.D, Woods J.W. and Crouch D.A. (1992). Direct evidence that primary acquired cell-mediated immunity is less resistant than is primary thymus-dependent humoral immunity to the depressive influence of wasting protein-energy malnutrition in weanling mice. Am. J. Clin. Nutr. 55, 1180-1185.

Woodward B. (1998). Protein, calories, and immune defenses. Nutr. Rev. 56, 84S-92S.

Accepted December 27, 2006